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**REMARKS**

Favorable reconsideration of the subject application, as amended above, is respectfully requested in view of the comments below.

Claims 9-29 are pending in the present application. Claims 10-12 and 19-29 were previously withdrawn from consideration; and claims 1-8 were previously canceled. Accordingly, claims 9 and 14-18 are presented for examination on the merits.

**I. Objection to Claim 18**

It is respectfully submitted that the objection to claim 18 is rendered moot by the amendment to the claim.

**II. Rejection of Claims 14-17 Under 35 U.S.C. § 112, Second Paragraph**

It is respectfully submitted that the amendments to the claims render this ground of rejection moot.

**III. Rejection of Claims 9 and 13-17 Under 35 U.S.C § 112, First Paragraph**

Claims 9 and 13-18 stand rejected under 35 U.S.C § 112, first paragraph. The Examiner states that the specification is enabling for enzymes having SEQ ID NO. 1, 3 and/or 5 and having a single specified substitution at position 251 and capable of hydrolyzing organophosphates. The Examiner asserts, however, that the specification does not enable any such enzymes encoded by polynucleotides having at least 80% homology to the polynucleotides encoding the above-referenced enzymes.

Applicants respectfully disagree with the Examiner's assertions.

It appears from the Examiner's remarks on pages 6-7 of the Office Action that the Examiner has confused the claimed polypeptide sequence (SEQ ID NO. 8), which is a recombinant *L. cuprina* enzyme, with the *M. domestica* sequence. To clarify, the claims are directed to the recombinant *L. cuprina* sequence (SEQ ID NO. 8) and sequences sharing 75% similarity thereto, which encodes organophosphate resistance.

The present claims are directed to recombinant enzymes having the specified activity (function), having a Leu, Ala, Ser, Ile, Val, Thr, Cys, Met or Gly residue at position 251 and which have at least about 75% sequence identity with SEQ ID NO. 8 (specified structure). The specification shows through sequence alignments that the claimed recombinant protein conferring malathion resistance can tolerate up to at least 25% sequence variation. The sequence alignments also demonstrate that it is essential for malathion resistance that the tryptophan at position 251 is replaced with a less bulky amino acid, i.e., Leu, Ser, Ala, Ile, Val, Thr, Met or Gly. Thus, the specification clearly demonstrates to one of skill in the art that numerous amino acid substitutions may be made while retaining the claimed enzyme activity. One of skill in the art at the time of the invention could readily introduce amino acid changes into the sequence based on the data provided in the specification, and expect to obtain functional enzymes having the claimed specificity, without undue experimentation. In particular, one of skill in the art at the time was capable of utilizing the disclosed alignment data to make conservative amino acid substitutions in the claimed recombinant enzyme.

Furthermore, the claimed recombinant enzyme is readily obtainable by performing nucleic acid hybridization with a probe having a sequence of any of the disclosed allele sequences under appropriate hybridization conditions, and expressing the protein and testing for activity as taught in the specification. One of skill in the art is capable of obtaining polynucleotide sequences encoding the claimed polypeptide. Indeed, claims to such polynucleotides encoding the enzyme have issued in US 6,235,515 (the parent application).

The specification teaches how to obtain the DNA encoding the claimed polypeptides, and teaches how to test for the claimed activity. The specification also provides several examples of recombinant enzymes as claimed, obtained from different species. Using the methods set forth in the specification, Applicants obtained several recombinant polypeptides having the claimed activity from different species and exhibiting up to 25 % amino acid variation.

Accordingly, the rejection of claims 9 and 13-18 under 35 U.S.C § 112, first paragraph is respectfully traversed.

#### **IV. Rejection of Claims 9 and 13-18 Under 35 U.S.C § 102(b)**

Claims 9 and 13-18 stand rejected under 35 U.S.C § 102(b) over Whyard et al., Pesticide Biochemistry and Physiology (Whyard a) or Whyard et al., Biochemical Genetics (Whyard b). The Examiner states that the cited prior art discloses a naturally occurring (non-recombinant protein) having the claimed sequence and activity.

This rejection is respectfully traversed as follows.

The enzyme that Whyard et al. isolated from *L. cuprina* is not the same as the organophosphate resistant recombinant enzyme of the present invention. Whyard et al. report that the  $K_m$  of the isolated enzyme towards malathion is about 11  $\mu M$  (See Figure 3 of Whyard

(a) and Table V of Whyard (b)). In contrast, the  $K_m$  of the presently claimed recombinant enzyme towards malathion is about  $1.09 \mu\text{M}$ . (See compound E3W251L at Table 2 of Devonshire et al, Pesticide Biochemistry and Physiology (2003) 76:1-13, copy enclosed). (Compound E3W251L is the recombinant enzyme of the present invention). Furthermore, the  $K_{cat}$  of the molecule described by Whyard (b) is  $45.7\text{min}^{-1}$  (See, for example, Table V of Whyard (b) ), whereas the  $K_{cat}$  of the presently claimed enzyme is shown to be  $220\text{min}^{-1}$  (See Table 2 of Devonshire et al.). Accordingly, the enzyme of the claimed invention has a  $K_m$  for malathion which is approximately ten-fold higher than that of the prior art, and a  $K_{cat}$  for malathion which is almost five-fold higher than that of the prior art. As a result, the recombinant enzyme of the claimed invention has almost a fifty-fold higher  $K_{cat}/K_m$  than that of the cited prior art (compare Table V of Whyard (b) with Table 2 of Devonshire *et al.*). Thus, it is clear that the isolated enzyme of the cited prior art is not the same as the recombinant enzyme of the claimed invention.

Moreover, as described by Whyard et al (a), the enzyme of the prior art was purified from both resistant and sensitive strains (See first two paragraphs of the abstract of Whyard (a)). As a result the authors concluded that the differences in malathion resistance and susceptibility between the two strains is due to a “quantitative rather than a qualitative change in the MCE of the two strains.” (see Abstract of Whyard (a)). In contrast, the present specification discloses that the malathion hydrolyzing activity of the insects analyzed is due to a **qualitative** difference in the enzymes possessed by resistant strains when compared to susceptible strains. This is highlighted in Table 2 of the present specification where all susceptible strains comprised a wild-type amino acid at position 251, whereas in resistant strains this amino acid was mutated.

Finally, Whyard (a) reported that the enzyme of the prior art is found, *inter alia*, in the mitochondria of both resistant and susceptible flies. In contrast, Applicant has advised that the enzyme of the present invention does not comprise a mitochondrial targeting signal, and is aware of no evidence that suggests that the enzyme of the claimed invention is found in the mitochondria of *L. cuprina*. Since a mitochondrial targeting sequence is an inherent property of an polypeptide sequence, it is clear that the enzyme of the prior art is not the same as the enzyme of the claimed invention.

In conclusion, there are three distinct lines of evidence that the enzyme reported by Whyard is not the same as the enzyme of the claimed invention. The enzyme of the claimed invention has i) a significantly higher  $K_m$  and  $K_{cat}$  towards malathion when compared to the enzyme of the prior art; ii) the enzyme of the claimed invention confers resistance through a qualitative change in the amino acid sequence of the esterase, whereas the resistance mechanism of the prior art enzyme is over-expression of the molecule; and iii) the molecule of the claimed invention has a different sub-cellular localization than that of the prior art molecule, due to the absence of a mitochondrial targeting sequence in the claimed enzyme. The totality of the evidence clearly demonstrates that the recombinant enzyme of the claimed invention is not inherently the same as the molecule of the cited prior art.

Accordingly, the rejection of claims 9 and 13-18 under 35 U.S.C. § 102(b) over the cited prior art is respectfully traversed.

It is respectfully submitted that the present application, as amended above, is in condition for allowance, an early notification thereof being earnestly solicited.

Serial No. 09/776,910

To the extent necessary, a petition for an extension of time under 37 C.F.R. § 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including extension of time fees, to deposit Account 500417 and please credit any excess fees to such deposit account.

Respectfully submitted,

MCDERMOTT, WILL & EMERY

A handwritten signature in black ink, appearing to read 'J. Toffenetti', written over the firm name.

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## Kinetic efficiency of mutant carboxylesterases implicated in organophosphate insecticide resistance

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### Abstract

Resistance to organophosphorus (OP) insecticides in *Lucilia cuprina* arises from two mutations in carboxylesterase E3 that enable it to hydrolyse the phosphate ester of various organophosphates, plus the carboxylester in the leaving group in the case of malathion. These mutations are not found naturally in the orthologous EST23 enzyme in *Drosophila melanogaster*. We have introduced the two mutations (G137D and W251L) into cloned genes encoding E3 and EST23 from susceptible *L. cuprina* and *D. melanogaster* and expressed them in vitro with the baculovirus system. The ability of the resultant enzymes to hydrolyse the phosphate ester of diethyl and dimethyl organophosphates was studied by a novel fluorometric assay, which also provided a sensitive titration technique for the molar amount of esterase regardless of its ability to hydrolyse the fluorogenic substrate used. Malathion carboxylesterase activity was also measured. The G137D mutation markedly enhanced (>30-fold) hydrolysis of both classes of phosphate ester by E3 but only had a similar effect on the hydrolysis of dimethyl organophosphate in EST23. Introduction of the W251L mutation into either gene enhanced dimethyl (23–30-fold) more than diethyl (6–10-fold) organophosphate hydrolysis and slightly improved (2–4-fold) malathion carboxylesterase activity, but only at high substrate concentration.

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**Keywords:** Kinetics; Malathion carboxylesterase; Organophosphorus hydrolase; Insecticide resistance

### 1. Introduction

The hydrolytic degradation of organophosphorus (OP) insecticides has long been implicated in the development of resistance to these compounds in treated insect populations [1]. One of the earliest such mechanisms proposed was the 'mutant aliesterase' in houseflies (*Musca domestica*), so-called because the flies lost the ability to hydrolyse certain aliphatic esters as they gained

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the ability to hydrolyse phosphotriesters [2]. A variation on this mutant aliesterase hypothesis was proposed to depend on a similar loss of aliesterase activity associated with the acquisition of an enzyme that hydrolyses both phosphotriesters and the carboxylester bonds in the side chain of malathion. This particular pattern was observed in both *M. domestica* [3–7] and a blowfly, *Chrysomya putoria* [8,9]. More recently both patterns of enzyme activity changes and cross-resistance profiles were observed in another blowfly, *Lucilia cuprina* [10], and in a number of other species [1]. The underlying molecular genetic changes have only recently been identified, initially in *L. cuprina* [11–13], and then in houseflies [14,15].

The enzymes implicated in both mechanisms belong to the carboxyl/cholinesterase multigene family of enzymes [16,17], many of which have been sequenced [18] and some of which have been crystallised and their 3D structures determined [19–21]. The key components of catalysis are therefore well defined and conserved across all members of the family, involving nucleophilic attack of a serine on the phosphorus or carbonyl carbon of the ester. A catalytic triad enabling a charge transfer through histidine from glutamate or aspartate enhances the reactivity of the serine. Other structural features are an 'oxyanion hole' and an 'acyl-binding pocket'. The former comprises small amino acids, typically two glycines and an alanine, that enable hydrogen bonding of the carbonyl oxygen of the ester to their NH groups in the peptide chain. The latter provides a hydrophobic region to accommodate the acid side chain of the ester when it binds to the serine [19,22].

The two resistance mechanisms ( $R_{OP-1}$  and  $R_{mal}$ ; [11–14]) described above in houseflies and blowflies are now known to depend on different mutations of a single gene, which in *L. cuprina* encodes the esterase E3. These mutations result in changes in the oxyanion hole and acyl-binding pocket regions of the esterase protein that alter its specificity for hydrolysing OPs and carboxylesters. The oxyanion hole mutation replaces a glycine by aspartate (E3G137D), which confers OP hydrolase activity, modest (7–20-fold) resistance to a range of diethyl OPs and slightly less resistance (4–12-fold)

to the corresponding dimethyl homologues [13,23]. It is proposed that the oxyanion hole aspartate activates a water molecule in an orientation favourable for nucleophilic attack on the phosphorus of the phosphorylated serine [12] but not on the carbonyl of bound carboxylesters. Both OP hydrolase and increased malathion carboxylesterase (MCE) activity are acquired when a tryptophan in the acyl-binding pocket is replaced by leucine (E3W251L), a smaller and less rigid peptide side chain [13] that is believed to create more space to accommodate the substrate and reduce the steric hindrance to the inversion that must occur about the phosphorus during hydrolysis. Mutations that alter the tryptophan to other small amino acids, serine or glycine, have also been identified recently in insects with high MCE activity [15,24]. Although the tryptophan to leucine mutation gives especially high resistance to malathion as a consequence of MCE activity, it also confers modest resistance to a wider range of OPs without carboxylester bonds. Resistance among the latter is generally higher for dimethyl OPs (5–27-fold) than for the corresponding diethyl homologues (2–6-fold; [23]). It is interesting to note, also, that increased expression of the orthologous gene (without the mutations) was found in diazinon resistant hornflies [25].

The glycine to aspartate and tryptophan to leucine mutations in *L. cuprina* E3 were shown unequivocally to generate the new catalytic functions by expressing the cloned genes in a baculovirus expression system and showing that the resulting enzymes had acquired activity against  $^{14}\text{C}$  chlorfenvinphos and had altered kinetics towards  $^{14}\text{C}$  malathion [12,13]. In quantitative terms however the chlorfenvinphos activity of E3G137D was not high, with  $K_m$  and  $k_{cat}$  estimated at  $126 \pm 21 \mu\text{M}$  and  $1.7 \text{ min}^{-1}$ , respectively. For comparison, Sabourault et al [26] recently obtained a  $k_{cat}$  of  $13.6 \pm 1.7 \text{ min}^{-1}$  for the wildtype CYP6A1 P450 from *M. domestica* against diazinon. Estimates of the  $K_m$  and  $k_{cat}$  of E3W251L for chlorfenvinphos were even lower, at  $21 \pm 2 \mu\text{M}$  and  $0.86 \text{ h}^{-1}$ , respectively, albeit good MCE activity was indicated by values of  $21 \pm 1 \mu\text{M}$  and  $43 \text{ min}^{-1}$  for malathion. There was no direct information on the OP hydrolase activity of this enzyme against malathion

but inhibition studies suggested it had sufficient activity to prevent malaoxon from irreversibly inhibiting MCE by enhancing hydrolysis of the dimethylphosphorylated enzyme.

We see thorough kinetic analyses of the mutant enzymes as crucial to understanding the biochemical basis of these resistance mechanisms. It is intriguing that what appears to be very modest detoxification kinetics should nevertheless confer major gene OP resistance on multiple species. In the case of OP resistant houseflies with the glycine to aspartate mutation, Sabourault et al. [26] have suggested that the acquisition of OP hydrolase activity may not even be the major molecular mechanism of resistance. Instead they suggest that resistance may be due more to some second order effect of the mutant enzyme that de-represses the expression of genes for other detoxifying enzymes such as the cytochrome P450s.

In order to clarify the role of the mutant esterases in OP detoxication and resistance, we have used more direct and sensitive fluorometric assays of OP hydrolase activity against diethyl and dimethyl OPs, and also re-assayed the MCE activities of the wild type and mutant enzymes using  $^{14}\text{C}$  malathion with higher specific activity. The glycine to aspartate and tryptophan to leucine mutations were analysed in *L. cuprina* E3 and also in the orthologous EST23 of *Drosophila melanogaster*, where as yet they have not been found in nature. We find that the G137D mutation confers OP hydrolase activity against both diethyl and dimethyl OPs in E3 and dimethyl OPs in EST23, while the W251L mutation more specifically confers activity against dimethyl OPs in both enzymes. The diethyl OP activity of E3G137D is significantly lower than that previously estimated by Campbell et al. [13] but we calculate that it is still sufficient to explain the diazinon resistance phenotype observed in *L. cuprina*. The MCE activity of E3W251L is 100-fold higher than that estimated by Campbell et al. [13] as a result of a 5-fold improvement in the measured value for  $k_{\text{cat}}$  and a 20-fold improvement in that for  $K_m$ . Together with the dimethyl OP hydrolase activity, its MCE activity readily accounts for the high levels of malathion resistance this mutation confers on *L. cuprina*.

## 2. Materials and methods

### 2.1. Enzyme constructs and expression

The G137D and W251L mutations were introduced into the cloned wild type (OP susceptible) alleles of the genes encoding E3 and EST23 by site-directed mutagenesis using the QuickChange kit (Stratagene). The resultant mutant genes were validated by sequencing and the validated sequence introduced to a baculovirus vector using the pFastBac1 system (Gibco-BRL Life Technologies). The eight enzymes made with the system were the wild type (WT) and W251L and G137D mutants of E3 and EST23 plus the double mutant (G137D/W251L) of E3, as well as a control virus with the  $\beta$ -glucuronidase (GUS) marker gene [27] inserted in the same bacmid cloning site. These eight enzymes were expressed in Sf9 cells as described previously [11,12], but using the HyQ SFX-insect serum free medium (HyClone), which gave better expression. Expression was optimised further by careful control of cell density at infection ( $3 \times 10^6$  viable cells  $\text{ml}^{-1}$ ) with a multiplicity of infection of two. Extracts were prepared by lysing cells at a concentration of  $10^8$  cells  $\text{ml}^{-1}$  in 0.1 M phosphate buffer pH 7.0 containing 0.05% Triton X-100 (rather than the previously used 0.5%, which interfered with assays for some other substrates to be reported separately). After a preliminary screen for 1-naphthyl acetate hydrolysis, those extracts with esterase activity were stored as 20–50  $\mu\text{l}$  aliquots at  $-80^\circ\text{C}$ , without removal of cell debris, and their esterase titre determined fluorometrically (see below).

### 2.2. Microplate assay for 1-naphthyl acetate hydrolytic activity

The efficiency of esterase expression was measured initially for all cell extracts from their 1-naphthyl acetate hydrolytic activity [28] adapted for microplates to allow comparison of widely differing (>1000-fold) activities. An aliquot of each extract was first diluted to the equivalent of  $10^7$  cells  $\text{ml}^{-1}$ . Up to eight extracts were then assayed simultaneously on a plate. A 20  $\mu\text{l}$  aliquot of the  $10^7$  cells  $\text{ml}^{-1}$  dilution was mixed with 20  $\mu\text{l}$  buffer

in the first well, and then serial 2-fold dilutions (20  $\mu$ l) made across the plate. 1-Naphthyl acetate (130  $\mu$ l, 0.3 mM in phosphate buffer pH 7.0) was added to all wells and incubated for 15 min at room temperature. The diazo blue coupling reagent (DBLS) was freshly prepared by dissolving 45 mg Fast Blue B salt (Sigma Cat. No. D3502) in 4.5 ml of water and adding 10.5 ml 5% sodium lauryl sulphate. DBLS (50  $\mu$ l) was added to each well, and the absorbance at 595 nm measured in a Biorad microplate reader after allowing the colour to develop fully in the dark for 20 min. Substrate and DBLS were dispensed with a multi-shot pipettor from low to high cell content wells to avoid the risk of enzyme carryover. Expression relative to cells transfected with the GUS virus was estimated by plotting the data as sigmoid curves and comparing the volumes of cell extract required to give an absorbance of 1.0.

### 2.3. Synthesis of *O, O*-dimethyl 4-methylumbelliferyl phosphate (dMUP)

Dimethylphosphorochloridate (1.6 g, 0.011 mol) in dry dimethylformamide (10 ml) was added to a suspension of the sodium salt of 4-methylumbelliferone (1.98 g, 0.01 mol) in the same solvent (10 ml) over 10 min, and the reaction stirred for a further 30 min. The product in dimethylformamide solution was decanted from the solid sodium chloride by-product, ethyl acetate (40 ml) was added, and extracted with solutions of sodium hydrogen carbonate (5%), then sodium chloride (20%), and dried over anhydrous sodium sulphate. Purification by chromatography on silica (20 cm  $\times$  2 cm column) eluting with 2% methanol in dichloromethane, gave 280 mg of colourless oil, which crystallised. The complete separation of product from traces of unreacted 4-methylumbelliferone was monitored on the column using a UV light. The product appeared as a dark blue band and the closely following reactant as a bright blue fluorescent band. The identity of the dMUP was confirmed by NMR spectrometry and its purity by TLC on silica in chloroform:acetone (1:1).  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were acquired on a Varian Inova 300 spectrometer. Chemical shift values were recorded in ppm using TMS as the internal reference.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$ 2.43

(d,  $J = 1.1$  Hz, 3H,  $\text{CH}_3$ ), 3.91 (d,  $J_{\text{P-H}} = 11.4$  Hz, 6H,  $\text{CH}_3\text{O}$ ), 6.26 (d,  $J = 1.1$  Hz, 1H,  $\text{H}_3$ ), 7.20 (s, 1H,  $\text{H}_8$ ), 7.22 (d,  $J = 8.2$  Hz, 1H,  $\text{H}_6$ ), 7.60 (d,  $J = 8.2$  Hz, 1H,  $\text{H}_5$ ).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$ 18.6 ( $\text{CH}_3$ ), 55.1 (d,  $J_{\text{P-C}} = 5.9$  Hz,  $\text{CH}_3\text{O}$ ), 108.4 (d,  $J_{\text{P-C}} = 5.6$  Hz,  $\text{C}_6$ ), 114.1 ( $\text{C}_3$ ), 116.2 (d,  $J_{\text{P-C}} = 4.7$  Hz,  $\text{C}_8$ ), 117.2 ( $\text{C}_{4a}$ ), 125.9 ( $\text{C}_5$ ), 151.8 ( $\text{C}_{8a}$ ), 152.8 (d,  $J_{\text{P-C}} = 6.1$  Hz,  $\text{C}_7$ ), 154.2 ( $\text{C}_4$ ), 160.3 ( $\text{C}_2$ ). A stock solution (10 mM in ethanol) was stored as aliquots at  $-20^\circ\text{C}$ .

### 2.4. *O, O*-Diethyl *O*-(2-oxo-2H-chromen-7-yl) phosphate (dECP)

The diethyl analogue of dMUP, but lacking the 4-methyl substituent and synthesised in the same way, was kindly provided by Sinead Walsh (IACR-Rothamsted, UK). Aliquots of a 10 mM stock solution in ethanol were stored at  $-20^\circ\text{C}$ .

### 2.5. Fluorometric assays

These were based on the release of the coumarin 'leaving group' from the organophosphorus inhibitors dMUP and dECP (Fig. 1), which was measured for 60 min at 3–5 min intervals in white 12  $\times$  8 well NUNC Polysorp fluoroplates using a BMG Fluorostar fluorometer (excitation 355 nm, emission 460 nm). Series of fluorescence readings from these continuous assays were stored to disc for subsequent analysis using Microsoft Excel. All assays were performed at  $25^\circ\text{C}$  and pH 7.0, which preserves esterase activity during the 30–60 min assays. Although this does not provide the ultimate sensitivity, because fluorescence is due to the coumarin anion ( $\text{pK}_a$  7.8) and so the product is only partially ionised at pH 7.0, the assay readily measured 1 pmol in a 100  $\mu$ l microplate assay. Careful control of pH was important for reproducibility because of the partial ionisation. The 'burst release' of fluorescence was used to titrate the molar amount of esterase in the cell extracts; others have used similar techniques with UV [29], fluorescent [30] or radioactive [28] reporter groups. This initial release of coumarin as the esterase catalytic site becomes dialkylphosphorylated occurs with a high bimolecular rate constant ( $k_2$ ), and any ensuing slow increase in fluorescence gives a measure of the hydrolysis

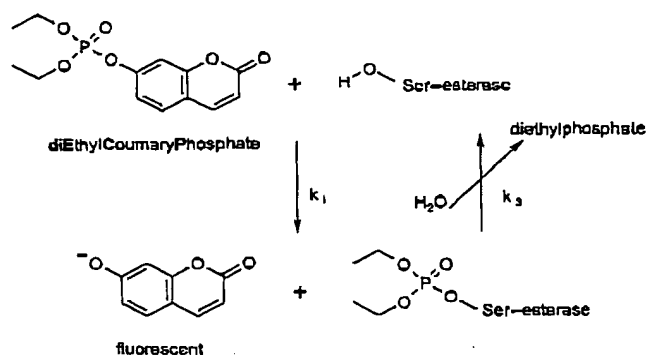


Fig. 1. Reaction scheme for titration of expressed esterases and measurement of  $k_{cat}$ . Note that dMUP differs from dECP only in that it is a dimethyl phosphate and has a methyl substituent on the 4-position of the coumarin.

rate of the inhibitor under conditions of enzyme saturation ( $k_{cat}$ , or  $k_3$  in Fig. 1). The latter rate depends solely on the nature of the dialkyl substituents on the phosphorus since the leaving group has already been released. The rates with dMUP and dECP therefore define the rate-limiting reaction for the slow hydrolysis of any dimethyl or diethyl OP ester, respectively. Although a radical mutation such as replacing the catalytic serine would abolish phosphorylation, all enzymes studied here interacted strongly with the inhibitors.

## 2.6. Titration of expressed esterases

Four 100  $\mu$ l reactions were set up for each expressed esterase in microplate columns 1–4: (A) a plate well blank containing 0.025% Triton X-100, 0.1 M phosphate buffer pH 7.0; (B) a substrate blank containing 100  $\mu$ M dECP in 0.025% Triton X-100, 0.1 M phosphate buffer, pH 7.0; (C) a cell blank containing 50  $\mu$ l cell extract mixed 1:1 with 0.1 M, phosphate buffer pH 7.0; and (D) a titration reaction containing 50  $\mu$ l cell extract mixed 1:1 with 0.1 M, phosphate buffer pH 7.0 containing 200  $\mu$ M dECP. All components except dECP (freshly prepared at a concentration of 200  $\mu$ M in buffer) were placed in the wells. Several enzymes were assayed simultaneously in a plate, and the reactions were started by adding dECP simultaneously to the second and fourth wells down a column using a multi-shot 8-tip pipettor with tips

only in positions 2 and 4. The interval to the first reading (typically 1 min) was noted for the subsequent calculations.

The mean value for the plate well blank (A) was subtracted from all readings before further calculations. Preliminary experiments with various cell extracts showed that they gave some fluorescence at 460 nm and that their addition to solutions of the assay products, 7-hydroxycoumarin or 4-methylumbelliferone, quenched fluorescence of these compounds by 39( $\pm$ 7)%. Fluorescence values in the titration reactions (D) were therefore corrected for this quenching effect after subtraction of the intrinsic fluorescence of the cell extracts (C). Finally, the substrate blank (B), taken as the mean from all the simultaneous assays in a plate, was subtracted to give the corrected fluorescence caused by the esterase-released coumarin. These corrections were most important for cell lines expressing esterase at very low level (<1 pmol/ $\mu$ l extract).

The fully corrected data were plotted as a progress curve, and the equilibrium slope extrapolated back to zero time to determine the amount of esterase, based on its stoichiometric interaction with the inhibitor (the 100  $\mu$ M concentration of dECP gave full saturation of the esterase catalytic sites of all these enzymes in 10–20 min). A calibration curve for 7-hydroxycoumarin was prepared alongside the reactions in all plates, and used to calculate molar concentration of enzyme and product formation.

### 2.7. Determination of esterase $k_{cat}$ with dimethyl and diethyl phosphates

Data from the same four titration assays above were used to measure the hydrolysis rate of the diethylphosphorylated esterases. Similar assays using dMUP as inhibitor and 4-methylumbelliferone (which had lower fluorescence than 7-hydroxycoumarin under the assay conditions) for calibration, gave the hydrolysis rate for the/dimethyl analogues. For some enzymes, the equilibrium slope was almost the same as the substrate blank, indicating no appreciable hydrolysis of the phosphorylated esterases, i.e., irreversible inhibition. However, some mutant enzymes gave a greater slope at equilibrium, indicating that the hydrolysis rate was significant. This rate, expressed as a fraction of the molar amount of esterase (calculated 'internally' from the burst release fluorescence with the same inhibitor) gives  $k_{cat}$ , the maximal rate of hydrolysis of diethyl or dimethyl OP compounds by the esterase. Any contribution from the small amount of endogenous esterase activity (see the GUS control) was minimised by using cell constructs that were expressing the inserted esterase gene at high levels (6–17-fold).

### 2.8. Malathion carboxylesterase (MCE) assay

MCE activity was assayed as described by Campbell et al. [13], but without diluting the specific activity of the  $^{14}\text{C}$  malathion ( $25\text{ mCi mmol}^{-1}$ ) for enzymes that appeared to have a low  $K_m$ . Activity was measured over the range 50 nM to 1  $\mu\text{M}$  to determine the  $K_m$  and  $k_{cat}$  for such enzymes (except for E3G137D which had negligible MCE activity), and analysed by non-linear regression using the Enzfitter 1.05 software (Elsevier-Biosoft), with graphical output to reveal any deviation from Michaelis–Menten kinetics.

## 3. Results and discussion

### 3.1. Esterase expression

Preliminary analysis of 1-naphthyl acetate hydrolysis by extracts of cells containing baculovirus-

esterase constructs (Fig. 2) identified those that expressed esterase activity from 10 to > 1000-fold that of the GUS control. The latter gave the same very low esterase activity as cells transfected with virus lacking any insert (data not shown). However, this rapid screening assay can be influenced by the mutations near the catalytic centre, which can potentially abolish (or enhance) enzyme activity even when the esterase protein is well expressed, so that it can only serve as a preliminary screen of successful expression. Fluorometric titration of the molar amount of esterase was therefore taken as the true measure of expression levels. All enzymes studied here, except EST23W251L, were from cells expressing the inserted esterase gene at a level at least 6-times the molar amount of titratable esterase in the control (Table 1). Even though expressed at only just over twice the control level, EST23 W251L was included for completeness. Work with various individual batches of a cell extract expressing at different levels (data not shown) has indicated that meaningful kinetic analysis can be achieved with such low expression without undue influence from the intrinsic esterase activity in the control.

### 3.2. Hydrolysis of phosphate esters

Previous analyses of these constructs have relied on a radiometric assay of diethylphosphate release from the hydrolysis of chlorfenvinphos [12,13]. Although this can provide data on the affinity ( $K_m$ )

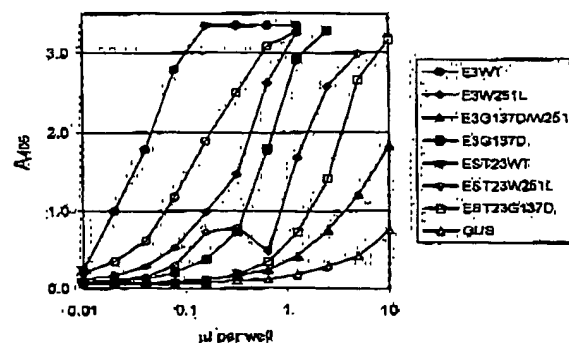


Fig. 2. 1-Naphthyl acetate hydrolysis by various concentrations of extracts of cells expressing baculovirus-esterase constructs.

Table 1  
Enzyme titres and  $k_{cat}$  values<sup>a</sup> for E3 and EST23 esterases and their mutants expressed in vitro

Enzyme	Titre ( $\mu\text{mol } \mu\text{l}^{-1}$ )	dECP $k_{cat}$ ( $\text{min}^{-1}$ )	dMUP $k_{cat}$ ( $\text{min}^{-1}$ )
GUS	0.4	0.0132	0
E3WT	$5.9 \pm 0.26$	$0.0009 \pm 0.0001$	$0.0018 \pm 0.0002$
E3G137D	$4.2 \pm 0.13$	$0.0500 \pm 0.0049$	$0.0570 \pm 0.0003$
E3W251L	$6.9 \pm 2.0$	$0.0092 \pm 0.0004$	$0.0610 \pm 0.0060$
E3G137D/W251L	$2.5 \pm 1.1$	$0.0210 \pm 0.0013$	$0.0530 \pm 0.0025$
EST23WT	$5.7 \pm 0.19$	$0.0010 \pm 0.0001$	$0.0009 \pm 0.0001$
EST23G137D	$4.0 \pm 0.99$	$0.0032 \pm 0.0003$	$0.0310 \pm 0.0005$
EST23W251L	0.9	$0.0060 \pm 0.0004$	$0.0210 \pm 0.0003$

<sup>a</sup> Except for GUS, all values are means ( $\pm$ SE) of 2–5 independent determinations.

of the enzymes for this particular OP substrate, as well as their turnover at saturating substrate ( $k_{cat}$ ), the latter is the rate-limiting step in the hydrolysis of these compounds, which depends on the hydrolysis of the relatively stable phosphorus-serine bond in the esterase. This can now be determined directly for all dimethyl and diethyl OPs by using the fluorogenic compounds dECP and dMUP, which has the advantage of measuring the rate of reaction and molar amount of enzyme within a single assay, rather than relying on widely differing techniques to measure these parameters independently [13]. However, the efficiency of an enzyme for 'purging' a compound in vivo at very low substrate concentration (defined by  $k_{cat}/K_m$ ) still requires knowledge of the affinity of the esterase for that particular compound.

The expressed E3WT (referred to as E3-OPS in some earlier publications) esterase from *L. cuprina* showed no appreciable activity with either dimethyl or diethyl OPs, whereas the two naturally occurring mutations enhanced hydrolysis of both types of compound (Fig. 3 and Table 1). The absolute magnitude of the increases is difficult to quantify because the basal activity in the WT enzyme was so low, and barely distinguishable from the GUS control, that accurate rate measurements were not possible. However, relative changes for different enzyme/substrate combinations could be made. With dimethyl substrates, the increase in activity was similar (approx. 30-fold) for both mutations, either alone or when combined in a single construct (Table 1), although this combination has not been found to occur naturally. The G137D mutation had a much greater effect (>50-fold) on the diethyl compounds than the W251L

mutation (10-fold), with the double mutant showing intermediate activity.

The G137D mutation is considered to act by introducing a negatively charged side chain in place of the original glycine in the oxyanion hole, a change that serves to hold a water molecule in a favourable position for nucleophilic attack on the phosphorus of the dialkylphosphorylated serine in the active site (12). The same mechanism is thought to underlie the enhancement of dephosphorylation seen when a histidine is introduced in place of the corresponding glycine in butyrylcholinesterase [31]; (O. Lockridge and C.A. Broomfield, pers. comm.). Such a mechanism should not be affected substantially by the size of the dialkyl substituents on the tetrahedral phosphorus, which are oriented away from the oxyanion hole. This is in accord with the broadly similar enhancement of  $k_{cat}$  for both substrates by the G137D mutation. The increase relative to the E3WT was, if anything, slightly greater for the diethyl than the dimethyl compound (Table 1), in line with the resistance patterns to the two classes of OP in bioassays [23].

The effect of the W251L mutation has been proposed to depend on replacement of the bulky aromatic group in the 'acyl binding pocket' by a less rigid and more accommodating branched aliphatic group, which might lower the energy of the transition state complex as the tetrahedral phosphorus undergoes inversion during hydrolysis of the serine-phosphorus bond [22]. Such a spatial constraint on the energy of this transition might be expected to impose itself more markedly on the diethylphosphorylated enzyme than on the smaller dimethyl homologue. However, the enhanced activity resulting from this mutation was much more

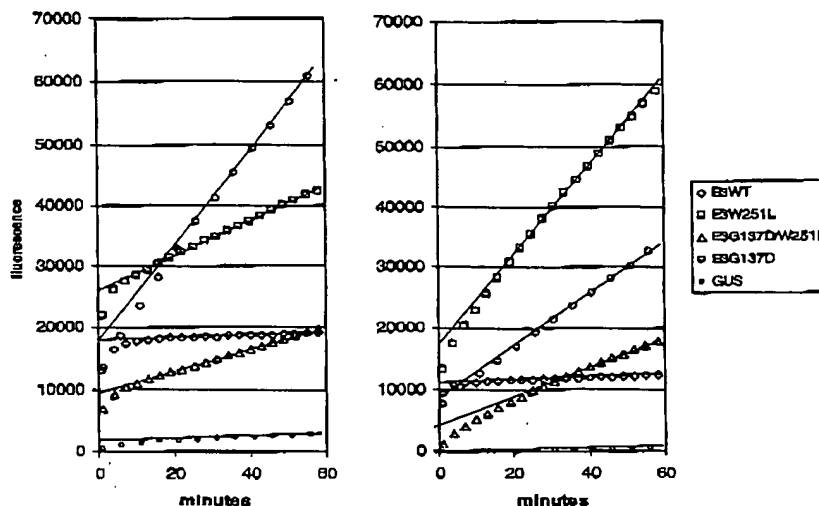


Fig. 3. Progress curves for the hydrolysis of dimethyl and diethyl OPs by cell extracts containing various *L. cuprina* E3 enzymes.

pronounced for dMUP than for dECP, indicating that the extra space created is still somewhat constraining on hydrolysis of the larger homologue. Indeed, both the WT and the mutant forms of the expressed esterases hydrolysed dMUP faster than dECP. By the same argument, it might be expected that the phosphorylation rate would be increased in the W251L mutant, but this was not apparent from the progress curves of coumarin release (Figs. 3 and 4). The greater activity with dMUP agrees well with the *in vivo* behaviour, in that flies carrying this mutation have 2–5-fold higher resistance to dimethyl OPs than to the corresponding diethyl analogues.

The introduction of both mutations together in the same construct had no additive effect on the hydrolysis of the dimethylphosphorylated enzyme, the individual or combined mutations all enhancing hydrolysis by about 30-fold. However, activity of the double mutant was intermediate with dECP, indicating that the enhanced activity provided by the G137D replacement was modulated by the spatial constraints on this larger homologue remaining after the W251L replacement. It is notable that crystal structure data for other carboxyl/cholinesterase enzymes phosphorylated by OP compounds have shown that only one of the alkyl groups is located in the acyl binding pocket, the

other being close to the space vacated by the leaving group [32,33]. We also note that a G137D/W251L double mutation has not yet been found to occur naturally in *L. cuprina* or *M. domestica*. Below we shall consider further the possible fitness effects of the double mutant.

The trends in our dECP and dMUP results are broadly in line with our published data using chlorfenvinphos [10,12–15] but the present  $k_{cat}$  values for the diethyl compounds are about an order of magnitude lower. This has been identified as due to a misunderstanding about the specific activity of the radiolabelled chlorfenvinphos used in the earlier work. Recalculations now indicate that the activities and kinetic parameters towards CVP in those papers should be taken as 20-fold lower.

As with the *L. cuprina* E3, the orthologous esterase EST23WT from *D. melanogaster* gave negligible hydrolysis of either substrate (Fig. 4). Introduction of the G137D or W251L mutations into EST23 enhanced its hydrolysis of dMUP to the same extent as with E3, but both had only a modest effect on dECP hydrolysis. This suggests that, with the larger ligand, the electronic and conformational benefits conferred by these mutations were compromised by other structural constraints in this enzyme, which shows an overall

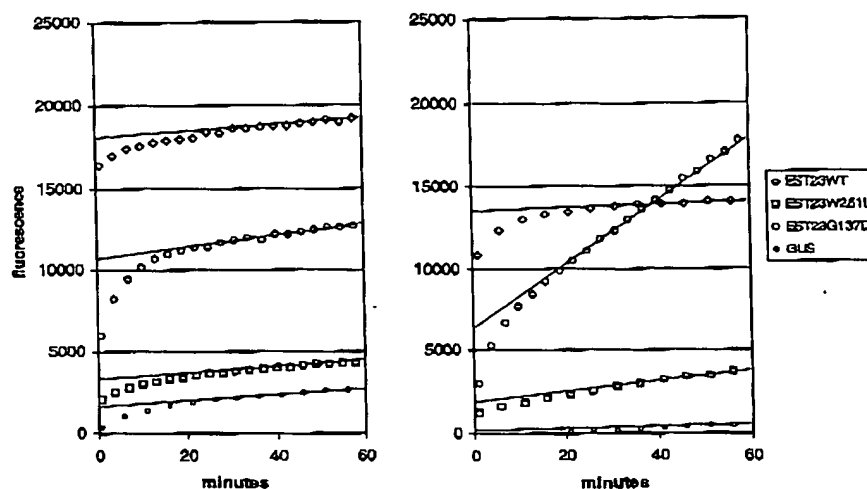


Fig. 4. Progress curves for the hydrolysis of dimethyl and diethyl OPs by cell extracts containing various *D. melanogaster* EST23 enzymes.

64% identity to E3 with all key residues in the catalytic site conserved. This may in part explain why the G137D mutation at least has not been found in EST23 in wild populations of *D. melanogaster*. OP resistance in this species appears to be due to insensitive acetylcholinesterase (AChE) target site mutations rather than enhanced hydrolysis [34,35]. However, Chen et al [35] attribute this to the lower affinity of OPs for EST23 than AChE in *D. melanogaster*, which is the reverse of the case for E3 and AChE in *L. cuprina*. Their argument can explain why neither G137D nor W251L has been found in EST23. The significance of the current result for EST23 is mainly the demonstration that the dECP hydrolysis due to the G137D change is contingent on other aspects of active site structure, which may be missing in even quite closely related enzymes. EST23 is only slightly less similar to E3 than is the orthologous aliesterase of *M. domestica*, in which G137D does confer diethyl OP resistance (64% versus 75% amino acid identity, [14]).

Campbell et al. [13] calculated that the mutant E3G137D esterase in *L. cuprina* has the capacity to detoxify the larger amount of OP applied to resistant insects. However, the 20-fold lower  $k_{cat}$  now found raises that issue again. This is especially pertinent in view of a proposal that the altered

enzyme (at least in *M. domestica*) is a 'loss of function' mutation that acts through the inability of the G137D form of the aliesterase to activate a repressor, so switching on a suite of genes encoding other detoxifying enzymes [26].

It is important to remember that the increased dose of insecticide applied to resistant insects need not be degraded *entirely* by the modified enzyme. Thus, a housefly, whether susceptible or resistant, has about 0.4 pmol of acetylcholinesterase, almost all of which must be inhibited to kill the insect [36]. The LD<sub>50</sub> of parathion or diazinon to susceptible *L. cuprina* is ca. 50 ng, or 200 pmol, so that only 0.2% of the applied dose of insecticide is required to bind, assuming an AChE content similar to that of houseflies. The remainder of the dose is rendered ineffective through detoxification (by metabolism, sequestering and partitioning into lipids), excretion and failure to penetrate. Since these processes are common to susceptible and resistant flies and will not be saturated at such doses [37,38], a similar proportion (99.8%) of the 20-fold higher dose of parathion (or 10-fold higher for diazinon) required to kill a resistant fly would also be removed without any need for a mutation that enhanced detoxification. With susceptible flies, this would not prevent the toxic oxon metabolite from reaching a sufficient concentration

for long enough in the vicinity of the AChE to inhibit the enzyme critically. For resistant flies to keep the paraoxon below this critical combination of concentration and time, metabolic capacity need only be enhanced sufficiently to remove most of the remaining 0.2% of the 4000 pmol LD50 of resistant flies, i.e., 8 pmol. This would be achieved within 3 h by 1 pmol of the E3G137D mutant esterase, which has a  $k_{\text{cat}}$  of  $0.05 \text{ min}^{-1}$ .

Approximately 0.1% of the total protein in a blowfly has been estimated to be E3 [13,39], which corresponds to about 30 pmol per fly, similar to that calculated for houseflies [40]. Thus, even if the E3 content were 30-fold less than this estimate, the enhanced OP hydrolase activity of the mutant enzyme would be sufficient to hydrolyse enough of the oxon. These calculations are broadly in line with the enhanced hydrolytic degradation measured over 3 h in susceptible and resistant blowflies treated with the susceptible LD50 of parathion [41].

Furthermore, the study by Hughes and Devonshire [41] showed that the hydrolysis of paraoxon is independent of NADPH (as was chlorfenvinphos hydrolysis in a separate study by Campbell et al. [13], indicating that it was not attributable to MFO activity. Since the E3G137D mutant esterase has the capacity to degrade the required amount of insecticide and so confer resistance in its own right, there is no need to invoke a secondary regulatory role for the mutation. However, MFO-mediated cleavage of parathion to diethyl phosphorothionate was also slightly greater in the OP resistant *L. cuprina* Q strain studied by Hughes and Devonshire [41] than it was in a susceptible strain. The strains had different

genetic backgrounds, although it was known that the Q strain had only the  $R_{\text{OP-1}}$  resistance locus, and the possibility of a common regulatory element for both biochemical mechanisms was raised, in line with the proposal of Sabaurault et al. [26]. The ideas in the latter paper revolve around the Rutgers housefly strain, which shows stronger resistance to diazinon (40-fold) as well as a broader spectrum of resistance than *L. cuprina* has to various classes of insecticide.

### 3.3. Malathion carboxylesterase activity

The overall effects of the mutations on MCE activity (Table 2) were similar to those reported earlier [13], although there are different contributions from changes in the affinity ( $K_m$ ) and turnover ( $k_{\text{cat}}$ ). The use of higher specific activity malathion in this study, together with a revised enzyme titration technique, resulted in more accurate determinations of the kinetic parameters. The E3WT now gave a much higher affinity ( $K_m$   $0.33 \mu\text{M}$  compared with  $200 \mu\text{M}$ ) and the E3W251L enzyme had a  $K_m$  of  $1.09 \mu\text{M}$ , in line with estimates made originally by Welling and Blaakmeer [7] for the MCE enzyme from houseflies. Compared with E3WT, the  $K_m$  of the E3W251L mutant for malathion was now 3–5-fold worse (higher), either alone or when combined with the G137D mutation in the same construct. This contrasts with a 20-fold improvement in  $K_m$  originally reported as a consequence of the very high value estimated for the E3WT. However, G137D alone had a strong adverse effect, increasing the  $K_m$  to ca.  $14 \mu\text{M}$ . In parallel with

Table 2  
MCE kinetics<sup>a</sup> for E3 and EST23 esterases and their mutants expressed in vitro

Enzyme	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\mu\text{M}^{-1} \text{min}^{-1}$ )
GUS	n/a <sup>b</sup>	n/a	n/a
E3WT	$0.33 \pm 0.08$	$54 \pm 6$	164
E3G137D	$13.8 \pm 4.4$	$7.8 \pm 0.6$	0.57
E3W251L	$1.09 \pm 0.4$	$220 \pm 17$	202
E3G137D/W251L	$1.5 \pm 0.2$	$5.4 \pm 1.2$	3.6
EST23WT	$0.60 \pm 0.07$	$96 \pm 4$	160
EST23G137D	n/a	n/a	n/a
EST23W251L	$1.28 \pm 0.02$	$178 \pm 15$	137

<sup>a</sup> All values are the means ( $\pm$ SE) of 2–5 independent determinations.

<sup>b</sup> n/a, no activity detectable.

these effects on affinity, the mutations had a marked effect on  $k_{\text{cat}}$ , the W251L mutation increasing it 4-fold to  $220 \text{ min}^{-1}$ . This is broadly in line with previous data [13] and with Welling and Blaakmeer's [7] estimate of  $55 \text{ min}^{-1}$  for housefly MCE, the net effect on the specificity constant ( $k_{\text{cat}}/K_m$ ) now being only a 25% increase. Thus in vivo, at very low malathion concentrations, the 'purging' capacity of MCE would be barely improved, but it would be 4-fold more efficient at high malathion concentrations. This effectively reverses the relative contributions of these components of hydrolysis compared to the earlier data [13].

The G137D mutation virtually abolished MCE activity through its adverse effects on both  $K_m$  and  $k_{\text{cat}}$  in both E3 and EST23. When combined in the same construct with W251L, malathion affinity was not affected but its maximal hydrolysis ( $k_{\text{cat}}$ ) was. It is notable that the carboxylester(s) of malathion has the 'opposite' orientation to that of OPs undergoing phosphate ester cleavage, since it has a small alcohol (ethyl) leaving group and a large 'acylating' group. Clearly, the entire acid moiety of malathion (including the ethylsuccinate and dimethylphosphate) could not be accommodated in the conventional acyl-binding pocket of carboxyl/cholinesterase enzymes, indicating that it must adopt a quite different orientation. Perhaps the "acyl-binding pocket" should be considered to constitute an "alkyl-binding pocket" in this particular context. This situation is similar to that discussed for the related juvenile hormone esterase [42], which has about 31% identity with E3 and Est23. Furthermore the carbonyl group in the aliphatic esters is planar, whereas the phosphorus in OPs has a tetrahedral configuration. Consequently, the approach of the water molecule during nucleophilic attack on the carbonyl will be at  $90^\circ$  to the carbon-serine bond, whereas it should be at  $180^\circ$  to this bond when approaching the phosphorus [22,43]. This could explain the contrasting effects of the G137D mutation on hydrolysis of the two types of ester.

Campbell et al. [13] did not have data for malaoxon hydrolysis, but inferred from inhibition experiments with this oxon analogue that recovery of the MCE activity of E3 following its inhibition by the malaoxon produced in vivo played a vital

role in maintaining the overall malathion degrading ability of the enzyme. The present data provide direct evidence to implicate this mechanism unequivocally. Indeed, the 4-fold catalytic improvement ( $k_{\text{cat}}$ ) in MCE activity conferred on E3 by the W251L mutation is small compared to its >30-fold enhanced malaoxon hydrolytic activity (as judged from the  $k_{\text{cat}}$  for dMUP hydrolysis), suggesting that this is the primary factor in maintaining MCE activity. However, hydrolysis of the carboxyl ester is clearly important in achieving the exceptional efficacy of the enzyme with malathion in comparison with other dimethyl OPs.

Although the  $K_m$  of the E3G137D/W251L double mutant is effectively as good as that of E3W251L, its  $k_{\text{cat}}$  for malathion is as poor as that of E3G137D (and about 50-fold worse than that of E3W251L). This is reminiscent of the double mutant's OP hydrolase activity, the  $k_{\text{cat}}$ s for which were not as high as those of E3G137D. Thus both the OP hydrolase activity of E3G137D and the MCE activity of E3W251L are compromised in the presence of the other mutation. This may in part explain why the double mutant has not as yet been reported in *L. cuprina* or *M. domestica*, albeit there might also have been insufficient time since OPs were first used for the appropriate intragenic recombination or second site mutation events to have occurred.

Although the absolute  $k_{\text{cat}}$  values of the respective enzymes are not the same, in relative terms the W251L mutation had a remarkably similar effect on MCE and dMUP activities in EST23 as it did in E3. Together with the evidence for W251L or equivalent mutations to Ser or Gly in malathion resistant *M. domestica* [15], it seems likely that this mutation can be a relatively versatile mechanism for evolving malathion detoxifying esterases in insects.

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